Functional CB1 cannabinoid receptors in human vascular endothelial cells

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Cannabinoid CB1 receptor mRNA was detected using reverse transcription–polymerase chain reaction (RT–PCR) in endothelial cells from human aorta and hepatic artery and in the ECV304 cell line derived from human umbilical vein endothelial cells. CB1 receptor-binding sites were detected by the high-affinity antagonist radioligand [^{125}I]AM-251. In ECV304 cells, both the highly potent synthetic cannabinoid agonist HU-210 and the endogenous ligand anandamide induce activation of mitogen-activated protein (MAP) kinase, and the effect of HU-210 was completely blocked, whereas the effect of anandamide was partially inhibited by SR141716A, a selective CB1 receptor antagonist. Transfection of ECV304 cells with CB1 receptor antisense, but not sense, oligonucleotides caused the same pattern of inhibition as SR141716A. This provides more definitive evidence for the involvement of CB1 receptors in MAP kinase activation and suggests that anandamide may also activate MAP kinase via an additional, CB1 receptor-independent, SR141716A-resistant mechanism. The MAP kinase activation by anandamide in ECV304 cells requires genistein-sensitive tyrosine kinases and protein kinase C (PKC), and anandamide also activates p38 kinase and c-Jun kinase. These findings indicate that CB1 receptors located in human vascular endothelium are functionally coupled to the MAP kinase cascade. Activation of protein kinase cascades by anandamide may be involved in the modulation of endothelial cell growth and proliferation.

Key words: anandamide, CB1 receptors, MAP kinase.

INTRODUCTION

Marijuana and its constituent cannabinoids are potent modulators of neurobehavioural functions, and they also have effects outside the central nervous system. Two G-protein-coupled receptors have been identified to date that recognize plant-derived cannabinoids as well as some endogenous lipid-like mediators, such as arachidonyl ethanolamide (anandamide) [1] and 2-arachidonyl glycerol [2,3]. CB1 receptors are located in the brain [4] and also in some peripheral tissues [5,6], whereas CB2 receptors are expressed by immune cells [7]. We have recently reported that plant-derived cannabinoids as well as anandamide elicit hypotension and bradycardia via peripherally located CB1 receptors [8]. Possible underlying mechanisms include presynaptic CB1 receptor-mediated inhibition of norepinephrine release from peripheral sympathetic nerve terminals [6,9,10], and/or direct vasodilation via activation of vascular cannabinoid receptors [8,11,12]. The latter may also be the target of endocannabinoids generated in circulating macrophages and platelets [13,14] or the vascular endothelium [15–18]. Vasoactive agents can dilate blood vessels by a direct action on vascular smooth muscle, or indirectly, via the vascular endothelium. CB1 receptors mediating vasodilation have been identified in cerebrovascular smooth muscle cells [12], and the presence of CB1 receptor mRNA in human umbilical vein endothelial cells was suggested by the results of reverse transcriptase–polymerase chain reaction (RT–PCR) experiments [16]. Cannabinoid-induced vasodilation has both endothelium-independent and endothelium-dependent components [18], and cannabinoids have been reported to modulate nitric oxide production [15] and calcium transients [19,20] in vascular endothelial cells. This suggests that CB1 receptors may also be present in the vascular endothelium, although in some blood vessels anandamide can also interact with a non-CB1 endothelial site to cause vasodilation [18,21]. Here we demonstrate through the use of RT–PCR, dideoxy sequencing and radioligand-binding techniques that CB1 receptors are expressed in different types of human vascular endothelial cells.

Several signalling pathways triggered by the activation of CB1 receptor have been described, including modulation of adenylate cyclase activity and N-type calcium channels. However, it is still a matter of debate whether cannabinoids can activate the mitogen-activated protein (MAP) kinase cascade through a G-protein-dependent pathway or via a non-cannabinoid receptor-mediated mechanism. By using the selective CB1 receptor antagonist SR141716A and CB1 receptor antisense oligonucleotides in cultured endothelial cells, we provide evidence that the synthetic cannabinoid ligand HU-210 activates the p44/42 MAP kinase pathway via CB1 receptors, whereas activation of MAP kinase by anandamide has both CB1-dependent and independent components. Furthermore, anandamide can also induce phosphorylation of the p38 and c-Jun kinases.

MATERIALS AND METHODS

Cells

Endothelial cells from normal human aortae and hepatic arteries were isolated and cultured as described [22]. The endothelial specificity of the cells was verified by the uptake of DiL-labelled acetylated low-density lipoprotein (DiL-Ac-LDL) [23]. ECV304 is a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord. Although recent unpublished information suggests that this cell line contains satellite markers of the T24 human bladder carcinoma cell line, the cells used in this study

Abbreviations used: JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; RT–PCR, reverse transcriptase–polymerase chain reaction.

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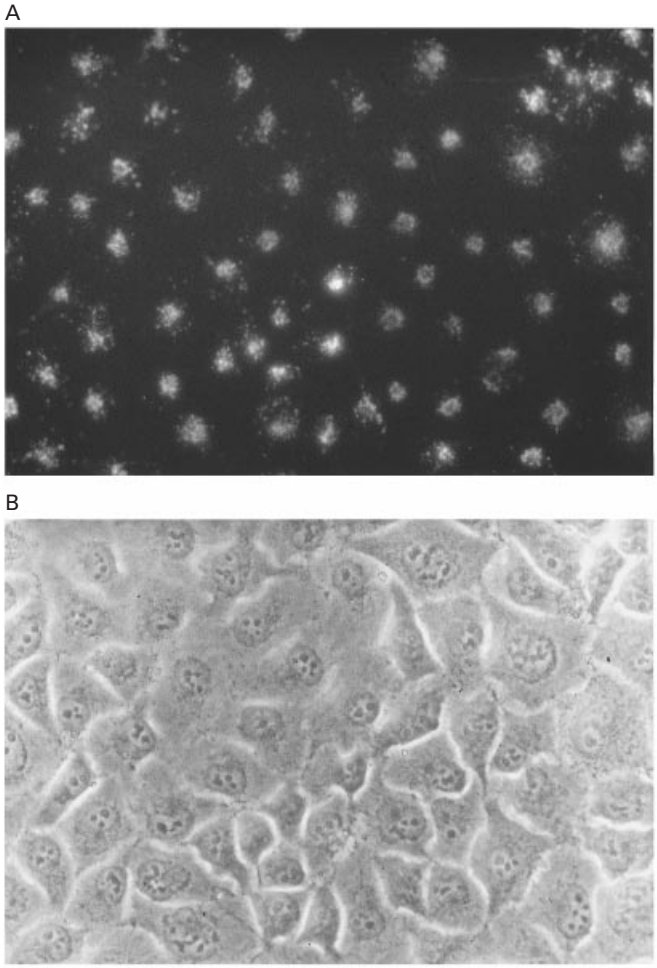

Figure 1 Uptake (internalization) of DiL-Ac-LDL by ECV304 cells

(**A**) Fluorescence micrograph of internalized DiL-Ac-LDL by ECV304 cells. (**B**) Phase contrast image of the micrograph in (**A**).

tested strongly positive for DiL-Ac-LDL uptake, a selective marker for vascular endothelial cells (Figure 1). The cells were cultured in 199 medium containing 10 % heat-inactivated fetal bovine serum under standard culturing conditions. EA.hy296 cells, another spontaneously transformed human umbilical vein endothelial cell line, were kindly provided by Dr Cora Edgell (University of North Carolina, Chapel Hill, NC, U.S.A.) and cultured according to [20].

RT–PCR

Total cellular RNA was isolated from confluent cultures of human endothelial cells by using the Snap Total RNA Isolation kit (Invitrogen). Five μg of total RNA was reverse-transcribed by random priming and incubation with 200 units of MMLV transcriptase at 37 °C for 1 h. The resulting single-stranded cDNA (5 μl) was then subjected to 30 cycles of PCR (Robocycler 96, Stratagene) under standard conditions. Samples were denatured at 95 °C for 5 min and, after the addition of the polymerase, subjected to 30 cycles of amplification each consisting of 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C, with a 7 min extension at 72 °C during the last cycle. Each PCR mixture (100 μl) contained the cDNA template, 1 μM of the primers, 200 μM of dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1 % Triton X-100 and 2.5 μM Taq polymerase (Promega). The primers used to amplify the human CB1 receptor gene [24] corresponded to the following sequences in transmembrane segment II: 5′-GCCTGGCGGTGGCAGACCTCC-3′ (sense) and transmembrane segment IV: 5′-GCAGCACGG-CGATCACAATGG-3′ (antisense). The human β-actin gene was also amplified as an internal control, using the primers: 5′-GTGGGGCGCCCCAGGCACCA-3′ (sense) and 5′-CTCCTT-AATGTCACGCACGATT-3′ (antisense). The expected size of the amplicons was 276 bp for the CB1 receptor and 500 bp for β-actin. The PCR products were electrophoresed on a 2 % agarose gel. RNA without reverse transcriptions did not yield any amplicons, indicating that there was no genomic DNA contamination.

Sequencing

The PCR amplified CB1 receptor gene fragment was purified by using the gene clean kit (Qiagen), and then inserted into the TA cloning vector. The cDNA was sequenced according to the dideoxynucleotide chain-termination method (Sequenase kit; United States Biochemicals).

Radioligand binding

The presence of CB1 receptor-binding sites on endothelial cells was tested by the specific binding of the high-affinity antagonist radioligand, [^{125}I]AM-251 [25]. Cells were harvested in phosphate-buffered saline containing 1 mM EDTA (pH 7.4) and centrifuged at 1000 ***g***. The cell pellet was homogenized in 5 ml of homogenization buffer (320 mM sucrose, 50 mM Tris/HCl, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.4). The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C, and the pellet was resuspended in fresh homogenization buffer and re-centrifuged twice more. The combined supernatants were centrifuged at 40000 ***g*** for 30 min, and the pellet was resuspended in 3 ml of assay buffer (50 mM Tris/HCl, 1 mM EDTA, 3 mM $MgCl_2$, pH 7.4) to yield a protein concentration of approx. 1 mg/ml. Binding was initiated by the addition of 50 μg of membrane protein to sialinized tubes containing 0.2 nM [^{125}I]AM-251 (2500 Ci/mmol) and a sufficient volume of assay buffer supplemented with 5 mg/ml BSA, to bring the total assay volume to 250 μl. Due to the low abundance of binding sites on cells and the limited number of cells available for assays, binding was tested at a single, submaximal concentration of the radioligand (0.2 nM), in the absence and presence of 20 μM SR141716A as unlabelled competitor. Triplicate aliquots were incubated at 30 °C for 1 h, followed by the addition of 2 ml of an ice-cold wash buffer (50 mM Tris/HCl, 1 mg/ml BSA, pH 7.4) and vacuum filtration through GF/B glass fibre filters. Filters were rinsed 3 more times with wash buffer, and the retained radioactivity was quantified by γ-radiation spectrometry.

Analysis of phospho-p44/42 MAP kinase, phospho-p38 MAP kinase and phospho-c-Jun N-terminal kinase (JNK) by Western immunoblotting

Phospho-p44/42 MAP kinase, phospho-p38 MAP kinase and phospho-JNK were quantified according to the protocol in a Western immunoblotting kit (BioLabs, New England). ECV304 cells were cultured in serum-free medium overnight prior to the addition of ligands, to reduce basal levels of MAP kinase phosphorylation. After treatment with different drugs, cells were washed at 4 °C with 1 × PBS, lysed in 100 μl of lysis buffer [26] and kept on ice for 10–15 min. Proteins were denatured by

boiling for 5 min, cooled on ice, and a 100 μg aliquot was fractionated by SDS/PAGE, using a 7.5 % gel. The separated fractions were electrotransferred on to a nitrocellulose membrane, blocked by incubation for 1–3 h in TPBS buffer (1 × PBS, 0.1 % Tween-20) containing 5 % (w/v) non-fat dry milk at room temperature. Blots were washed 3 times with TPBS buffer, then incubated with primary antibody (at 1:1000 dilution in TPBS) with gentle agitation overnight at 4 °C. The membranes were washed again 3 times, incubated with anti-rabbit secondary antibody (1:2000) in blocking buffer, and gently agitated for 2 h at room temperature. Bands were visualized using an ECL detection system.

DNA mobility shift assay (DMSA)

DMSA and preparation of nuclear extracts for DMSA were carried out as described previously [27].

Treatment of cells with CB1 receptor sense and antisense oligodeoxynucleotides

Phosphorothioate and phosphodiester oligodeoxynucleotides were synthesized as previously described [28]. CB1 receptor sense or antisense oligonucleotides were sterilized by centrifugation through 0.2 μm Centrex cellulose acetate filters. ECV304 cells were maintained as monolayers in 6-well plates in 199 medium containing 10 % heat-inactivated fetal bovine serum. Cells were washed 3 times with Opti-MEM (Gibco–BRL) pre-warmed to 37 °C. To each well, 100 μl of Opti-MEM containing 4 μl of lipofectin and oligonucleotides (1 μM as final concentration) was added. After incubation for 4 h at 37 °C, the medium was replaced with the appropriate cell growth medium containing 1 μM of the same oligonucleotide, and the cells were incubated for an additional 48 h at 37 °C under an atmosphere of 5 % CO_2. Following oligonucleotide treatment, cells were stimulated with anandamide or HU-210.

Stable CB1 receptor transfections

Cell lines expressing the human CB1 cannabinoid receptor were established by cloning the coding region of the human CB1 receptor gene into the pcDNA3 mammalian expression vector and transfecting it into 293 cells by the lipofectin technique (Gibco–BRL). Stable transformants were selected using growth medium containing G418 (0.4 mg/ml). Colonies were picked up and allowed to expand, then tested for receptor binding. Transfected cell lines were maintained in Dulbecco's modified Eagle's medium with 10 % FBS plus 0.4 mg/ml G418.

RESULTS

Expression of the CB1 receptor gene in human endothelial cells

As shown in Figure 2, RT–PCR using endothelial cells from human aorta, hepatic arteries or ECV304 cells produced single discrete bands of the same, expected size, while no band was obtained using EA.hy296 cells. Similar strong bands were obtained for β-actin from all four groups of cells. Direct sequencing of the amplified DNA obtained from all three cell types confirmed its perfect homology with the sequence of the corresponding 276-nucleotide-long segment of the human CB1 receptor gene. To test whether the receptor mRNA is effectively translated into receptor protein, radioligand-binding assays were done in separate batches of hepatic artery endothelial cells. Specific binding was reproducibly detected (5.8 ± 2.2 fmol/mg, $n = 3$).

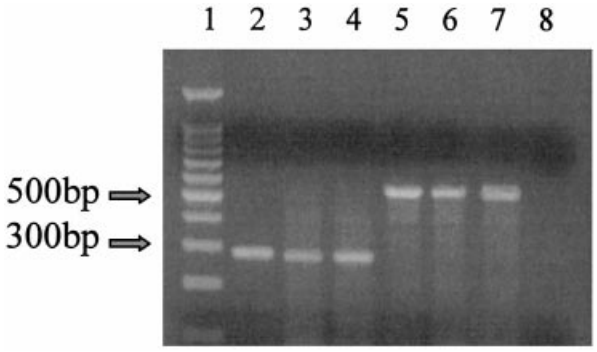

Figure 2 RT–PCR analysis of CB1 receptor and β-actin gene expression in human vascular endothelial cells

RT–PCR was performed using total RNA from endothelial cells from hepatic artery (lanes 2 and 5), aorta (lanes 3 and 6) and umbilical vein (lanes 4 and 7), using specific primers for the human CB1 receptor gene (transmembrane segments II and IV, lanes 2–4) and the human β-actin gene (lanes 5–7). Negative control was without cDNA (lane 8).

CB1 receptor signalling in ECV304 cells

In ECV304 cells, both HU-210 (100 nM) and anandamide (10 μM) were found to activate p42/44 MAP kinase. As these concentrations correspond to $\sim$ 50 times K_d for each ligand, the effects produced are expected to be near-maximal. Time-course studies illustrated in Figure 3 indicated that peak levels appeared within 5 min after the addition of anandamide, with a rapid decline of activity thereafter. MAP kinase activation developed more slowly in response to HU-210 treatment, when peak levels were observed between 10 and 30 min. The effect of HU-210 was blocked in the presence of 1 μM SR141716A, a selective CB1

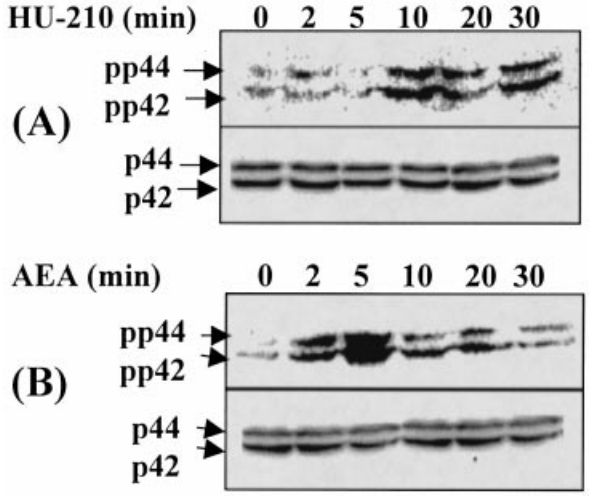

Figure 3 Time-course of activation of p42/44 MAP kinase by HU-210 and anandamide

Serum-starved ECV304 cells were incubated for the indicated time periods with 100 nM HU-210 or 2.5 μM anandamide (AEA). Western blot analysis of the phosphorylated MAP kinase isoforms was performed as described in the Materials and methods section.

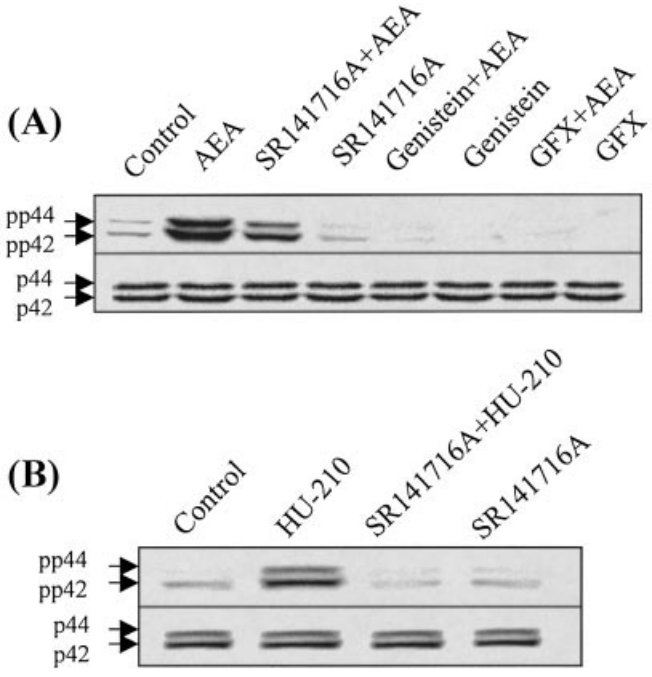

Figure 4 Mechanism of anandamide (A) and HU-210 (B) activation of p42/44 MAP kinase in ECV304 cells

(**A**) Anandamide induction of phospho-p42/44 MAP kinase is partially inhibited by SR141716A and blocked by the tyrosine kinase inhibitor genistein or the protein kinase C inhibitor GFX. Serum-starved cells were preincubated for 30 min with 50 μM genistein or 2 μM GFX, or for 50 min with 2.5 μM SR141716A, prior to the addition of 2.5 μM anandamide (AEA) or vehicle for another 5 min, as indicated. (**B**) HU-210 induction of phospho-p42/44 MAP kinase is blocked by SR141716A. Serum-starved cells were preincubated for 50 min with 2.5 μM SR141716A, prior to the addition of 100 nM HU-210 for another 10 min. Phospho-p42/44 MAP kinase was detected by Western blotting.

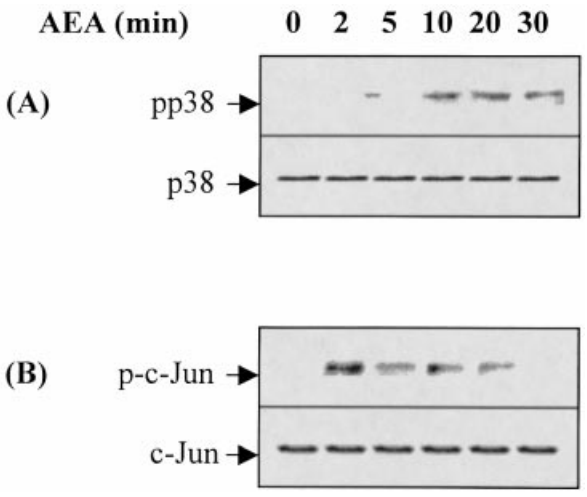

Figure 5 Time-course of anandamide induction of phospho-p38 kinase (A) and phospho-c-Jun kinase (B)

Western blotting analysis was performed as described in the Materials and methods section.

receptor antagonist (Figure 4B), while the effect of anandamide was only partially inhibited by the same concentration of SR141716A (Figure 4A). Furthermore, MAP kinase activation

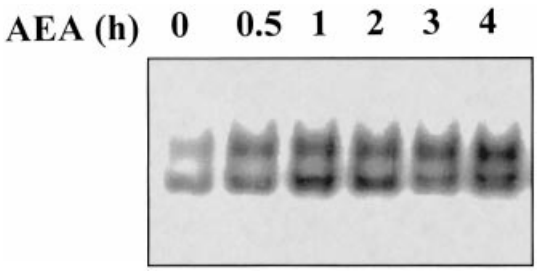

Figure 6 Anandamide-induced time-dependent activation of AP1 in ECV304 cells

Serum-starved ECV304 cells were incubated with 2.5 μM anandamide (AEA) for the indicated time periods. DNA mobility shift assay was performed using 10 μg of crude ECV304 cell extract and ^{32}P-labelled AP1 consensus oligonucleotide as probe.

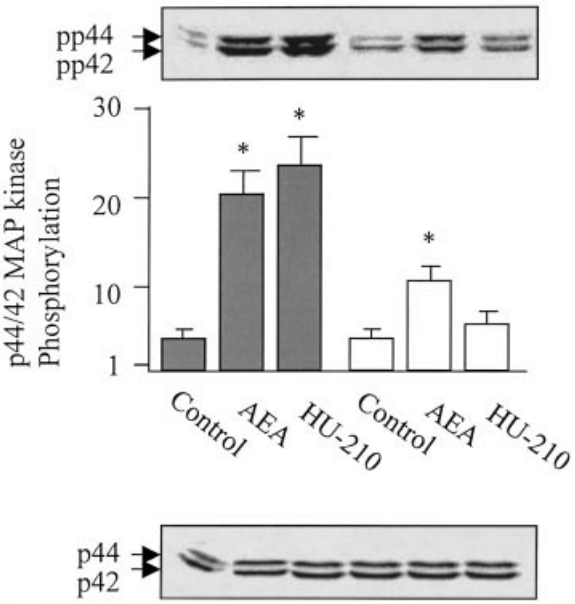

Figure 7 Anandamide and HU-210 induction of phospho-p42/44 MAP kinase in ECV304 cells transfected with CB1 receptor sense or antisense oligonucleotides

ECV304 cells were transfected with a CB1 receptor sense (■) or an antisense (□) oligonucleotide using the lipofectin method, and then incubated for 48 h at 37 °C. The serum-starved transfected cells were then incubated for 5 min with vehicle (lane 1) or 2.5 μM anandamide (AEA, lane 2), or for 10 min with 100 nM HU-210 (lane 3). *significant difference ($P < 0.05$) from corresponding control value.

by anandamide appeared to require the activation of a genistein-sensitive tyrosine kinase as well as protein kinase C (PKC), because treatment of ECV304 cells with genistein (50 μM) or the PKC inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide ('GFX') (2 μM) completely abrogated the effect of anandamide on MAP kinase activity (Figure 4A).

Anandamide was also found to activate p38 kinase and c-Jun kinase in ECV304 cells (Figure 5). A possible downstream target of anandamide signalling via these kinases is the AP1 transcription factor, which was strongly induced by anandamide. Activation of AP1 mRNA was evident within 30 min and peaked at 4 h following treatment with 2.5 μM anandamide (Figure 6).

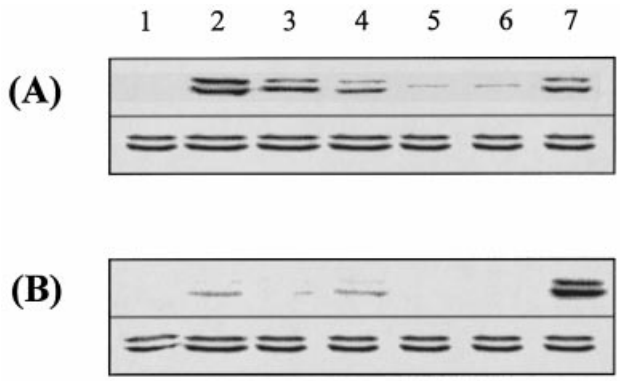

Figure 8 Anandamide and HU-210 induction of phospho-p42/44 MAP kinase in 293 cells stably transfected with the CB1 receptor cDNA (A) and in mock-transfected 293 cells (B)

Cells were treated with vehicle (1), 2.5 μM anandamide (AEA, 2), 100 nM HU-210 (3), 2.5 μM AEA + 2.5 μM SR141716A (4), 2.5 μM SR141716A (5), 2.5 μM SR141716A + 100 nM HU-210 (6) and 1 μM epidermal growth factor (7).

The inhibition of MAP kinase by CB1 receptor antisense oligonucleotide in ECV304 cells

To confirm the role of CB1 receptors in the observed effects, ECV304 cells were preincubated for 24 h with CB1 receptor sense or antisense oligonucleotides (Figure 7). In cells preincubated with CB1 receptor sense oligonucleotides, both anandamide (2.5 μM) and HU-210 (100 nM) elicited strong activation of p42/44 MAP kinase, which was similar or greater than that observed in control ECV304 cells. However, in cells preincubated with CB1 receptor antisense oligonucleotides, HU-210 failed to activate MAP kinase, and activation of MAP kinase by anandamide was markedly reduced (Figure 7). Since the effects seen in the cells preincubated with antisense oligonucleotides were similar to those observed in control cells in the presence of SR141716A, it is plausible that they reflected a decline of functional CB1 receptors due to the inhibition of the translation of CB1 receptor mRNA.

CB1 receptor-mediated activation of p42/44 MAP kinase in stably transfected 293 cells

To further test the role of CB1 receptors in signalling via MAP kinase activation, we stably expressed the human CB1 receptor in 293 cells that do not normally express such receptors. Stably transfected 293 cells contained 35.3 fmol/mg protein of specific CB1 receptor-binding sites, while mock-transfected cells had no detectable binding. As illustrated in Figure 8(A), HU-210 and anandamide activated p44/42 MAP kinase in stably transfected 293 cells. The activation by HU-210 was completely blocked and activation by anandamide was partially inhibited by SR141716A. In contrast, in mock-transfected 293 cells neither of the two cannabinoids elicited activation of MAP kinase, although epidermal growth factor was able to cause very strong MAP kinase activation (Figure 8B).

DISCUSSION

The results presented provide multiple lines of evidence for the presence of functional CB1 cannabinoid receptors in human vascular endothelial cells. RT–PCR using cDNA from both primary cultured and transformed endothelial cells yielded amplicons of the expected size, which extends previous findings in umbilical vein endothelial cells [16]. In the present study, the identity of these amplicons was further verified by their perfect sequence homology with the corresponding segment of the human CB1 receptor gene. Ligand-binding experiments using an iodinated radioligand with subnanomolar affinity for CB1 receptors demonstrated the presence of specific binding sites on endothelial cells from human hepatic arteries, which indicates that the CB1 receptor gene is not only transcribed, but the message is translated into protein with a functional binding site. The absence of CB1 receptor message or binding in EA.hy296 cells indicates that not all endothelial cells express CB1 receptors, and is also compatible with the results of a recent study in EA.hy296 cells, in which the relatively high concentrations of SR141716A required to inhibit anandamide-induced calcium transients implicated a receptor other than the CB1 receptor in that effect [20].

The results of additional experiments indicate that endothelial CB1 receptors are coupled to the MAP kinase signalling cascade. Both the endogenous ligand anandamide and the highly potent synthetic cannabinoid HU-210 were found to activate the p42/44 MAP kinase in ECV304 cells, and the effect of HU-210 was completely blocked, whereas the effect of anandamide was partially inhibited by the CB1 receptor antagonist SR141716A. Although SR141716A may also inhibit cannabinoid effects mediated via a non-CB1 receptor mechanism [18,21], results with antisense oligonucleotides provide more definitive evidence for the involvement of CB1 receptors in MAP kinase activation. Preincubation of ECV304 cells with a CB1 receptor antisense, but not sense, oligonucleotide caused the same pattern of inhibition as SR141716A, i.e. complete block of MAP kinase activation by HU-210 and partial inhibition of the response to anandamide (Figure 7). Together, these observations strongly suggest that both HU-210 and anandamide activate MAP kinase in ECV304 cells via CB1 receptors, which is in agreement with the reported involvement of CB1 receptors in MAP kinase activation in a number of other cell types and non-neuronal tissues [29–32]. They also indicate, however, that anandamide can also activate MAP kinase via an additional, CB1 receptor-independent, SR141716A-resistant mechanism, which is similar to the non-CB1 receptor-mediated activation of MAP kinase by anandamide in murine lymphoid (B9) and myeloblastic (FDC-P1) cell lines [33]. Anandamide has been reported to interact, possibly as a low-affinity endogenous ligand, with a number of sites other than cannabinoid receptors, which often do not recognize plant-derived or synthetic cannabinoids. Such sites include the dihydropyridine [34,35] or phenylalkylamine-binding sites [36] of L-type Ca^{2+} channels, vanilloid VR1 receptors [37,38], Shaker-related voltage-gated K^+ channels [39], or putative endothelial receptors that mediate mesenteric vasodilation in response to anandamide but not to other cannabinoids [18,21]. Whether a similar site(s) may contribute to the CB1 receptor-independent activation of MAP kinase by anandamide in ECV304 cells is unclear. However, the putative endothelial receptor for anandamide identified in rat mesenteric arteries is unlikely to be involved, as the vasodilation mediated by this receptor was found to be susceptible to inhibition by SR141716A [18,21].

There is growing evidence to indicate that G-protein-coupled receptors activate not only classical signalling pathways resulting in short-term biological responses, but also trigger nuclear signalling via tyrosine kinase cascades, such as the MAP kinase pathway, to influence gene expression related to cell growth and proliferation. Cytokine-induced activation of endothelial MAP kinase has been shown to be involved in increased endothelial

cell migration [40] and proliferation [41,42], whereas p38 kinase activation has been linked to reorganization of the microfilament network [41,43], increased vascular cell-adhesion molecule 1 ('VCAM1') expression [44], cell migration [45] and angiogenesis [41,46]. The role of c-Jun kinase in endothelial cell functions is less well characterized, although there is some evidence that it mediates inhibition of DNA synthesis and it may be involved in endothelial cell apoptosis [47,48]. The present finding that all three kinases can be activated by cannabinoids should stimulate further studies into the possible role of endocannabinoids in modulating endothelial growth and proliferation, including angiogenesis.

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